AD_____

CONTRACT NO: DAMD17-91-C-1140

TITLE: LIMITED TOXICITY AND MUTAGENICITY TESTING OF FIVE UNICHARGE PROPELLANT COMPOUNDS

SUBTITLE: Evaluation of Five Unicharge Propellants in the Ames/ Salmonella Plate Incorporation Assay

PRINCIPAL INVESTIGATOR: Vincent B. Ciofalo, Ph.D.

CONTRACTING ORGANIZATION: Pharmakon Research International, Inc.
P.O. Box 609
Waverly, PA 18471

REPORT DATE: December 6, 1991

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 6, 1991	Final Report (9/20/91 - 12/31/91)

4. TITLE AND SUBTITLE

LIMITED TOXICITY AND MUTAGENICITY TESTING OF FIVE UNICHARGE PROPELLANT COMPOUNDS

5. FUNDING NUMBERS

Contract No.
DAMD17-91-C-1140

6. AUTHOR(S)

Vincent B. Ciofalo, Ph.D.
Leon F. Stankowski, Jr., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Pharmakon Research International, Inc.
P.O. Box 609
Waverly, PA 18471

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Subtitle: Evaluation of Five Unicharge Propellants in the Ames/Salmonella Plate Incorporation Assay

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

n-Methyl-, n-ethyl- and n-butyl-2-nitratoethyl nitramine (Me, Et, and BuNENA), and bis-(2,2-dinitropropyl) acetal/formal (~50/50 mixture; ±diphenyl amine stabilizer; BDNPA/F ±DPA), were tested in the Ames/Salmonella assay in strains TA1535, TA1537, TA1538, TA98 and TA100 up to a maximum of 10,000 µg/plate or a toxic limit with and without metabolic activation (±S9). All samples induced reproducible, significant, dose-dependent, 2.6- to 20-fold increases in revertant frequencies in strain TA1535 +S9. Similar 1.6- to 10-fold increases were observed for MeNENA, EtNENA and BDNPA/F ±DPA in TA1535 -S9. Significant and/or dose-dependent increases, to 1.3- to 3.1-fold control values, were reproducibly observed in TA100 for BDNPA/F ±DPA ±S9, and for EtNENA +S9. All other sample/strain/S9 combinations produced negative results. On the basis of results in strain TA1535 with S9, the rank order of mutagenic potential is EtNENA > BuNENA > BDNPA/F +DPA = BDNPA/F -DPA >> MeNENA.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Ames/Salmonella assay; unicharge propellants; methyl-NENA, ethyl-NENA, butyl-NENA, bis-(2,2-dinitropropyl) acetal, bis-(2,2-dinitropropyl) formal, RA III, lab animals	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__(a)__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Accession For	
NTIS GRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

PI - Signature / Date

TABLE OF CONTENTS

Evaluation of Five Unicharge Propellants in the Ames/Salmonella Plate Incorporation Assay

PH 301-US-001-91
PH 301-US-002-91
PH 301-US-003-91
PH 301-US-004-91
PH 301-US-005-91

SUMMARY

Five unicharge propellant compounds were evaluated in the Ames/Salmonella Plate Incorporation Assay to determine their ability to induce reverse mutations at selected histidine loci in five tester strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98 and TA100) in the presence and absence of an exogenous metabolic activation system (S9). Toxicity of each compound was first evaluated in a prescreen by treating duplicate cultures of strains TA1538 and TA100 with each test article at doses of 50.0, 167, 500, 1670 and 5000 µg/plate in the absence of S9.

Based upon results of the toxicity prescreens, MeNENA and EtNENA initially were evaluated in the mutation assay in all five tester strains at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with and without S9. BuNENA was evaluated concurrently at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with S9, and 50.0, 167, 500, 1670, 3330 and 5000 µg/plate without S9. DNPA/F ±DPA initially were evaluated in the mutation assay in all five tester strains at doses of 16.7, 50.0, 167, 500, 1000 and 1670 µg/plate with and without S9. Six dose levels of each compound were evaluated with and without S9 in the event of unacceptable toxicity or insolubility at the highest dose levels evaluated. The S9 mixture included 6% (v/v) Aroclor 1254-induced male Sprague-Dawley rat liver homogenate with the appropriate buffer and cofactors. However, statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.4 to 19-fold control values, were observed in at least one tester strain with and/or without S9, for each test article. Therefore, MeNENA, EtNENA, and DNPA/F ±DPA were re-evaluated in confirmatory assays under identical conditions. BuNENA was re-evaluated in a confirmatory assay at doses of 16.7, 50.0, 167, 500, 1670 and 5000 µg/plate with and without S9.

All five test articles reproducibly induced statistically significant, dose-dependent increases in revertant frequencies, to approximately 2.6- to 20-fold control values, in tester strain TA1535 with S9. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.6- to 10-fold control values, also were reproducibly observed for MeNENA, EtNENA, and DNPA/F ±DPA in strain TA1535 without S9. In addition, statistically significant and/or dose-dependent increases in revertant frequencies, to approximately 1.3- to 3.1-

SUMMARY (continued)

fold control values, also were reproducibly observed in strain TA100 for bis-DNPA/F ±DPA with and without S9, and for EtNENA with S9. Similar, but unconfirmed, increases in revertant frequencies, to approximately 1.4- to 1.8-fold control values were observed for MeNENA (TA100 +S9), EtNENA (TA98 -S9), BuNENA (TA1535 -S9), DNPA/F +DPA (TA98 -S9), and DNPA/F -DPA (TA98 +S9). These latter unconfirmed increases, however, are considered to be statistical aberrations due to random fluctuation of the spontaneous revertant frequencies. All positive and negative control values in all assays were within acceptable limits.

Therefore, the results for all five test articles were positive in the Ames/Salmonella Plate Incorporation Assay under the conditions, and according to the criteria, of the test protocol. On the basis of the results observed in strain TA1535 with S9, the rank order of mutagenic potential (revertants per μmol/plate) is EtNENA > BuNENA > DNPA/F +DPA = DNPA/F -DPA >> MeNENA.

STUDY DESCRIPTIVE

Sponsor:	U.S. Army Medical Research and Development Laboratory Fort Detrick Frederick, MD 21702-5010
Study Numbers:	PH 301-US-001-91 PH 301-US-002-91 PH 301-US-003-91 PH 301-US-004-91 PH 301-US-005-91
Date Protocol Signed:	September 23, 1991
Date Assay Initiated:	September 30, 1991
Date Assay Completed:	October 31, 1991
Pharmakon Reference:	Notebook #1452: page 146 #1491: pages 177, 350 #1516: pages 1-249, 249A
Study Monitor:	Major Nathaniel Powell, U.S. Army Medical Research and Development Laboratory
Study Director:	Leon F. Stankowski, Jr., Ph.D., Pharmakon Research International, Inc.
Technical Performance:	Leon F. Stankowski, Jr., Ph.D., Teressa A. Polinsky, M.S., and Diane M. Messina, A.A.

PURPOSE AND RATIONALE

This assay measures the ability of a test article to induce reverse mutations at specific histidine loci in various tester strains of Salmonella typhimurium (Ames, et al., 1975; Maron and Ames, 1983; Maron, et al., 1981). Five different Salmonella tester strains (TA1535, TA1537, TA1538, TA98 and TA100) are used to evaluate each test article over a wide range of concentrations in the presence and absence of an exogenous metabolic activation system (S9). Chemicals capable of inducing mutations have been demonstrated to increase the frequency of histidine revertants in selected Salmonella tester strains in the presence and/or absence of S9.

TEST ARTICLES

All test articles were received by Pharmakon Research on September 20, 1991 in clear glass containers. n-methyl-2-nitratoethyl nitramine (MeNENA; CAS: 17096-47-8; Lot #XAP-MeNENA-6B), n-ethyl-2-nitratoethyl nitramine (EtNENA; CAS: 85068-73-1 Lot #XAP-EtNENA-4B) and n-butyl-2-nitratoethyl nitramine (BuNENA; CAS: 82486-82-6 Lot #XAP-BuNENA-15B) were provided as preweighed, single-use samples, and were described as a white solid, a yellow liquid, and a yellow liquid, respectively. MeNENA contained 30% added water for transport. Mixtures of bis-(2,2-dinitropropyl)acetal (CAS: 5108-69-0 and bis-(2,2-dinitropropyl) formal (CAS: 5917-61-3), with and without diphenyl amine stabilizer (DNPA/F ±DPA; +DPA, "Set #1"; -DPA, "Set #2"), also were described as yellow liquids. Information regarding technical aspects of the test article, as provided by the sponsor, was recorded in the sponsor's file. For the purposes of this study, the test articles were stored at room temperature in the containers received from the sponsor. At the time of testing the test articles exhibited the same physical characteristics as noted upon arrival. There was no apparent change in the physical states of the test articles during storage.

EtNENA, BuNENA, DNPA/F +DPA, and DNPA/F -DPA were used directly as received. However, samples of MeNENA were uncapped and placed in a desiccator (with desiccant) for approximately 24 hours prior to use, to remove the added water. All required dilutions were made with dimethyl sulfoxide (DMSO), Lot #902873, supplied by Fisher Scientific (Fairlawn, NJ). Dilutions were prepared the day of the test and used within two hours of preparation.

TEST SYSTEM

Test Organism/ Strains:	Salmonella typhimurium - TA1535, TA1537, TA1538, TA98 and TA100
Source:	Dr. Bruce N. Ames University of California Biochemistry Dept. Berkeley, California 94720

All strains contain a uvrB deletion mutation (affecting DNA excision repair), as well as an rfa mutation (affecting membrane permeability). In addition, strains TA98 and TA100 contain the plasmid pKM101, which enhances the error-prone DNA repair system normally present in this organism. Strains TA1535 and TA100 detect base pair substitution mutations affecting the hisG locus. In contrast, strains TA1538 and TA98 detect frameshift mutations affecting the hisD locus, while TA1537 detects frameshift mutations at the hisC locus. All tester strains were checked for the presence of the appropriate genetic markers on approximately a monthly basis.

Test Cultures

Fresh cultures for mutagenesis testing were prepared by quickly thawing a vial of frozen working stock cultures of each tester strain and transferring the culture to 25 ml of Oxoid Nutrient Broth #2. After growth for approximately 6 hours at 37°C in an orbital shaking incubator, samples of each culture were diluted 1:4 in distilled water and optical densities were determined at 650 nm. Cultures with optical densities of 0.40 to 0.60 (approximately 1-2 x 10^9 cells/ml; representative of cells in late exponential or early stationary phase) were utilized for this study.

Control Articles

Triplicate cultures of each strain were evaluated with the appropriate solvent in the presence and absence of S9 to serve as negative solvent controls. In order to validate the responsiveness of the test system, triplicate cultures of each tester strain were evaluated with known positive control chemicals. Positive controls evaluated in the absence of S9 were specific for each strain and included: TA1535 and TA100 - sodium azide (10.0 µg/plate; Sigma Chemical Company, Lot #56C-0263); TA1537 - 9-aminoacridine (150 µg/plate; Sigma Chemical Company, Lot #56C-0231) and TA1538 and TA98 - 2-nitrofluorene (5.00 µg/plate; Aldrich Chemical Company, Lot #2610PE). 2-Anthramine (2.50 µg/plate; Sigma Chemical Company, Lot #33F-0816) was evaluated in all five tester strains in the presence of S9.

TOXICITY PRESCREENS

Toxicities of the test articles were determined in preliminary toxicity prescreens by evaluating the growth of the background lawn and/or frequency of spontaneous revertants. The test articles were evaluated at doses of 50.0, 167, 500, 1670 and 5000 µg/plate in the absence of S9 (page 18). Each test article dose, as well as the appropriate solvent control, was evaluated in duplicate cultures in strains TA1538 and TA100. However, the DNPA/F ±DPA coalesced from solution/formed oily droplets at doses ≥500 µg/plate.

Treatment with Test and Control Articles

Treatment was performed by combining 0.1 ml tester strain, 0.1 ml of the appropriate concentration of the test article or solvent and 2 ml of molten top agar (supplemented with 0.5mM histidine/0.5mM biotin). The tubes were vortexed and the mixture was poured onto minimal glucose plates, evenly distributed, and allowed to solidify. Within an hour the plates were inverted and incubated in the dark at 37°C for 48 hours. All cultures/plates were identified using computer-generated adhesive labels that

included information regarding study number, date, strain, test/control article, and ±S9.

Scoring

Following the 48-hour incubation, the background lawn and spontaneous revertants were scored for normal, inhibited or no growth. Inhibited growth was characterized by the absence of a confluent bacterial lawn and/or the presence of pindot colonies.

MUTATION ASSAYS

Salmonella which have undergone reversion to his^+ form colonies in the absence of histidine. In contrast, his^- Salmonella can only undergo a limited number of doublings (due to the histidine supplement in the top agar) and form the typical background lawn. Mutation assays were performed in triplicate cultures in all five tester strains for each test article dose, as well as positive and solvent controls. Following incubation for 48 hours, revertant colonies were enumerated with an automated colony counter.

MeNENA and EtNENA initially were evaluated in the mutation assay in all five tester strains at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with and without S9. BuNENA was evaluated concurrently at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with S9, and 50.0, 167, 500, 1670, 3330 and 5000 µg/plate without S9. DNPA/F ±DPA initially were evaluated in the mutation assay in all five tester strains at doses of 16.7, 50.0, 167, 500, 1000 and 1670 µg/plate with and without S9. However, statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.5- to 19-fold control values, were observed in at least one tester strain with and/or without S9, for each test article. Therefore, MeNENA, EtNENA, and DNPA/F ±DPA were re-evaluated in confirmatory assays under identical conditions. BuNENA was re-evaluated in a confirmatory assay at doses of 16.7, 50.0, 167, 500, 1670 and 5000 µg/plate with and without S9 (doses evaluated in the confirmatory assay were adjusted based upon toxicity observations made in the original assay).

Treatment with Test and Control Articles

Treatment for the mutation assay was performed exactly as described in the toxicity prescreen, except that the test and control articles were evaluated in triplicate cultures in all five tester strains in the presence and absence of an exogenous metabolic activation system (S9). Cultures treated in the presence of S9 contained 0.5 ml of the S9 mixture. The S9 mixture contained 8mM $MgCl_2$, 33mM KCl, 4mM NADP, 5mM glucose-6-phosphate, 100mM Na_2HPO_4 (pH 7.4) and 6% (v/v) Aroclor 1254-induced male Sprague-Dawley rat liver homogenate.

Bacterial Contaminant Evaluation

To ensure the sterility of solvents, compounds and equipment, standard contamination evaluations were performed with each assay. The solvent, top agar, S9 mix, and highest dose of the test article were evaluated at the same volumes used in the assay. The test article, solvent and S9 mix were evaluated as in the mutation assay, but in the absence of added Salmonella. Top agar was also plated alone on minimal glucose plates. All plating was done in triplicate. Plates were incubated for 48 hours at 37°C and then scored for bacterial growth.

Scoring

Revertant colonies were enumerated on an Artek electronic colony counter interfaced with an IBM PC/AT computer for data acquisition. Solvent and positive controls were scored first, and test article treated cultures were scored only if the average negative control values were within historical ranges ($\bar{x} \pm 2SD$; see below). A summary of the results is presented in the Summary Data Tables (pages 21-30).

Historical Data - Spontaneous Revertants*

Strain	S9	n	Average(± 1SD)	Range($\bar{x} \pm 2SD$)
TA1535	-	173	9.65 ± 2.78	4.08 - 15.2
	+	176	10.3 ± 2.80	4.67 - 15.9
TA1537	-	172	7.87 ± 2.54	2.79 - 12.9
	+	171	9.50 ± 2.75	4.00 - 15.0
TA1538	-	177	5.92 ± 2.41	1.11 - 10.7
	+	178	13.5 ± 3.60	6.26 - 20.6
TA98	-	186	19.0 ± 4.92	9.14 - 28.8
	+	195	27.4 ± 6.77	13.9 - 40.9
TA100	-	184	83.8 ± 15.9	52.0 - 116
	+	188	96.9 ± 16.0	64.8 - 129

*January 1, 1990 - September 30, 1991

Evaluation Criteria

A positive result is defined as a statistically significant, dose-dependent increase in the number of histidine-independent revertants with at least one dose level inducing a revertant frequency that is two-fold the spontaneous solvent control value. Statistical analyses were performed using the program developed by Snee and Irr (1981), with significance established at the 95%

confidence limit. If the test article does not induce a statistically significant, dose-dependent increase in revertant frequency, but does induce a revertant frequency at one dose level that is two-fold the spontaneous control value, the result is considered equivocal. A negative result is defined as the absence of a statistically significant or dose-dependent increase in the number of histidine-independent revertants.

Statistical analyses (summarized in Table 2, page 19) generally are performed only when a 50% increase in revertant frequency, relative to the concurrent negative controls, is observed. This 50% "trigger" was selected based upon the normal, spontaneous variation observed among replicate negative control cultures (see above), as well as spontaneous fluctuation observed in this laboratory among groups of cultures treated with a variety of test article judged to be negative in this assay. However, statistical analyses also were performed in a limited number of cases where smaller (but apparently dose-dependent) increases were observed, or where such 50% increases were observed in the original, but not the confirmatory, mutation assay.

Comparisons of mutagenic potential of the five test articles were made using the uniformly positive results observed in strain TA1535 with S9. The slopes of the dose-response curves were re-calculated for the initial linear portion of the response (by simple linear regression on the untransformed data), corrected for molecular weight, and used to rank-order the five test articles.

Records Maintained

All correspondence pertinent to the study between the sponsor and Pharmakon Research International, Inc., the protocol, raw data, test article dispensation reports, quality assurance reports and the final report are maintained in the Pharmakon Archives.

Good Laboratory Practice Statement

Except that analytical analyses of dosing solutions were not performed to verify the accuracy or stability of the test article dosing solutions, this study was conducted in compliance with the Good Laboratory Practice Regulations for non-clinical laboratory studies as developed by the U.S. Food and Drug Administration (21 CFR, Part 58), the Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing Chemicals (ISBN 92-64-12221-4), and the U.S. Environmental Protection Agency (40 CFR, Parts 160 and 792). There were no other deviations from the GLP Regulations which affected the quality or integrity of the study. Q.A.U. findings derived from the inspections during the conduct of the study and from the audit of the final report are documented and have been provided to the study director and the test facility management.

RESULTS AND DISCUSSION

Five unicharge propellant compounds were evaluated in the Ames/Salmonella Plate Incorporation Assay to determine their ability to induce reverse mutations at selected histidine loci in five tester strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98 and TA100) in the presence and absence of an exogenous metabolic activation system (S9). Toxicity of each compound was first evaluated in a prescreen by treating duplicate cultures of strains TA1538 and TA100 with each test article at doses of 50.0, 167, 500, 1670 and 5000 µg/plate in the absence of S9 (page 18). All five test articles were then evaluated in the mutation assay in triplicate cultures in strains TA1535, TA1537, TA1538, TA98 and TA100 in the presence and absence of S9. Six dose levels of each compound were evaluated with and without S9 in the event of unacceptable toxicity or insolubility at the highest dose levels evaluated. The S9 mixture included 6% (v/v) Aroclor 1254-induced male Sprague-Dawley rat liver homogenate with the appropriate buffer and cofactors. However, statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.4- to 19-fold control values, were observed in at least one tester strain with and/or without S9, for each test article. Therefore, all five test articles were re-evaluated in confirmatory assays under identical (or similar) conditions.

MeNENA

Results of the toxicity prescreen indicated n-methyl-2-nitratoethyl nitramine (MeNENA) was not toxic to either strain at doses of 50.0, 167, 500, 1670 and 5000 µg/plate. In addition, the test article was freely soluble at all doses evaluated. Based upon these findings, MeNENA was evaluated in the mutation assay in all five tester strains at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with and without S9 (page 21). Normal growth was again observed for all strains at all doses of MeNENA with and without S9, and the test article again was freely soluble at all doses evaluated. Revertant frequencies for all doses of MeNENA in strains TA1537, TA1538, TA98 and TA100 with and without S9 approximated or were less than those observed in the concurrent negative control cultures. In contrast, statistically significant, dose-dependent increases in revertant frequencies, to approximately 2.6- to 1.6-fold control values, were observed in strain TA1535 with and without S9, respectively. MeNENA was re-evaluated in a confirmatory assay under identical conditions, and similar results were observed (page 22). Revertant frequencies for all doses of MeNENA in strains TA1537, TA1538 and TA98 with and without S9, and in strain TA100 without S9, approximated or were less than control values. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 13- to 2.0-fold control values, were observed in strain TA1535 with and without S9, respectively. In addition, a

statistically significant, dose-dependent increase in revertant frequencies, to approximately 1.4-fold control values, was observed in strain TA100 with S9.

EtNENA

Results of the toxicity prescreen indicated n-ethyl-2-nitratoethyl nitramine (EtNENA) was not toxic to either strain at doses of 50.0, 167, 500, 1670 and 5000 µg/plate. In addition, the test article was freely soluble at all doses evaluated. Based upon these findings, MeNENA was evaluated in the mutation assay in all five tester strains at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with and without S9 (page 23). Inhibited growth (characterized by a reduced background lawn and/or the presence of pindot colonies) was observed in tester strains TA1538 and TA100 at doses ≥5000 µg/plate with S9, and in strains TA98 and TA100 at doses of 5000, 7500 and/or 10,000 µg/plate without S9. The test article again was freely soluble at all doses evaluated. Revertant frequencies for all doses of EtNENA in strains TA1537, TA1538 and TA98 with S9, and in strains TA1537, TA1538 and TA100 without S9, approximated or were less than those observed in the concurrent negative control cultures. In contrast, statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.6- to 19-fold control values, were observed in strain TA1535 with and without S9, and in strains TA98 and TA100 without S9. EtNENA was re-evaluated in a confirmatory assay under identical conditions, and similar results were observed (page 24). Revertant frequencies for all doses of EtNENA in strains TA1537, TA1538 and TA98 with and without S9, and in strain TA100 without S9, approximated or were less than control values. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.3- to 20-fold control values, were observed in strains TA1535 and TA100 with S9, and in strain TA1535 without S9.

BuNENA

Results of the toxicity prescreen indicated n-butyl-2-nitratoethyl nitramine (BuNENA) was not toxic to either strain at doses of 50.0, 167 and 500 µg/plate. Inhibited growth was observed in both tester strains at doses of 1670 and 5000 µg/plate. In addition, the test article was freely soluble at all doses evaluated. Based upon these findings, BuNENA was evaluated in the mutation assay in all five tester strains at doses of 167, 500, 1670, 5000, 7500 and 10,000 µg/plate with S9, and 50.0, 167, 500, 1670, 3330 and 5000 µg/plate without S9 (page 25). Inhibited growth was again observed in all tester strains at doses of 500, 1670, 5000, 7500 and/or 10,000 µg/plate with S9, and at doses of 1670, 3330 and/or 5000 µg/plate without S9. The test article again was freely soluble at all doses evaluated. Revertant frequencies for all doses of BuNENA in strains TA1537, TA1538, TA98 and TA100 with and without S9 approximated or were

less than those observed in the concurrent negative control cultures. In contrast, statistically significant, dose-dependent increases in revertant frequencies, to approximately 6.8- to 1.6-fold control values, were observed in strain TA1535 with and without S9, respectively. BuNENA was re-evaluated in a confirmatory assay at doses of 16.7, 50.0, 167, 500, 1670 and 5000 µg/plate with and without S9 (doses evaluated in the confirmatory assay were adjusted based upon toxicity observations made in the original assay; page 26). Inhibited growth was again observed in all tester strains at doses of 500, 1670 and/or 5000 µg/plate with and/or without S9. Revertant frequencies for all doses of MeNENA in strains TA1537, TA1538, TA98 and TA100 with and without S9, and in strain TA1535 without S9, approximated or were less than control values. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 3.5-fold control values, were observed in strain TA1535 with S9.

DNPA/F -DPA

Results of the toxicity prescreen indicated bis-(2,2-dinitropropyl) acetal/formal without DPA stabilizer (DNPA/F -DPA) was not toxic to either strain at doses of 50.0 and 167 µg/plate. Inhibited growth was observed in both tester strains at doses of 500, 1670 and 5000 µg/plate. In addition, the test article coalesced from solution/formed oily droplets at doses ≥500 µg/plate. Based upon these findings, DNPA/F -DPA was evaluated in the mutation assay in all five tester strains at doses of 16.7, 50.0, 167, 500, 1000 and 1670 µg/plate with and without S9 (page 27). Except for strain TA1538 with S9, inhibited growth was again observed in all tester strains at doses of 1000 and/or 1670 µg/plate with and/or without S9. The test article again was incompletely soluble at doses ≥500 µg/plate. Revertant frequencies for all doses of DNPA/F -DPA in strains TA1537 and TA1538 with S9, and strains TA1537, TA1538 and TA98 without S9, approximated or were less than those observed in the concurrent negative control cultures. In contrast, statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.5- to 5.9-fold control values, were observed in strains TA1535, TA98 and TA100 with S9, and strains TA1535 and TA100 without S9. DNPA/F -DPA was re-evaluated in a confirmatory assay under identical conditions, and similar results were observed (page 28). Revertant frequencies for all doses of DNPA/F -DPA in strains TA1537, TA1538 and TA98 with and without S9 approximated or were less than control values. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.7- to 9.0-fold control values, were observed in strains TA1535 and TA100 with and without S9.

DNPA/F +DPA

Results of the toxicity prescreen indicated bis-(2,2-dinitropropyl) acetal/formal with DPA stabilizer (DNPA/F +DPA)

was not toxic to either strain at doses of 50.0, and 167 µg/plate. Inhibited growth was observed in both tester strains at doses of 500, 1670 and 5000 µg/plate. In addition, the test article coalesced from solution/formed oily droplets at doses ≥500 µg/plate. Based upon these findings, DNPA/F +DPA was evaluated in the mutation assay in all five tester strains at doses of 16.7, 50.0, 167, 500, 1000 and 1670 µg/plate with and without S9 (page 29). Inhibited growth was again observed in all tester strains at doses of 1000 and/or 1670 µg/plate with and/or without S9. The test article again was incompletely soluble at doses ≥500 µg/plate. Revertant frequencies for all doses of DNPA/F +DPA in strains TA1537, TA1538 and TA98 with S9, and strains TA1537 and TA1538 without S9, approximated or were less than those observed in the concurrent negative control cultures. In contrast, statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.6- to 6.8-fold control values, were observed in strains TA1535 and TA100 with S9, and in strain TA1535 without S9. Statistically significant or dose-dependent increases in revertant frequencies, to approximately 1.4- to 1.8-fold control values, also were observed in strains TA98 and TA100 without S9. DNPA/F +DPA was re-evaluated in a confirmatory assay under identical conditions, and similar results were observed (page 30). Revertant frequencies for all doses of DNPA/F +DPA in strains TA1537, TA1538 and TA98 with and without S9 approximated or were less than control values. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.5- to 10-fold control values, were observed in strains TA1535 and TA100 with and without S9.

CONCLUSIONS

All five test articles reproducibly induced statistically significant, dose-dependent increases in revertant frequencies, to approximately 2.6- to 20-fold control values, in tester strain TA1535 with S9. Statistically significant, dose-dependent increases in revertant frequencies, to approximately 1.6- to 10-fold control values, also were reproducibly observed for MeNENA, EtNENA, and DNPA/F ±DPA in strain TA1535 without S9. In addition, statistically significant and/or dose-dependent increases in revertant frequencies, to approximately 1.3- to 3.1-fold control values, also were reproducibly observed in strain TA100 for DNPA/F ±DPA with and without S9, and for EtNENA with S9. Similar, but unconfirmed, increases in revertant frequencies, to approximately 1.4- to 1.8-fold control values were observed for MeNENA (TA100 +S9), EtNENA (TA98 -S9), BuNENA (TA1535 -S9), DNPA/F +DPA (TA98 -S9), and DNPA/F -DPA (TA98 +S9). These latter unconfirmed increases, however, are considered to be statistical aberrations due to random fluctuation of the spontaneous revertant frequencies. All positive and negative control values in all assays were within acceptable limits.

Therefore, the results for all five test articles were positive in the Ames/Salmonella Plate Incorporation Assay under the conditions, and according to the criteria, of the test protocol. On the basis of the results observed in strain TA1535 with S9, the rank order of mutagenic potential (revertants per μmol/plate) is EtNENA > BuNENA > DNPA/F +DPA = DNPA/F -DPA >> MeNENA (page 20).

REFERENCES

Ames, B.N., J. McCann, and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/Microsome Mutagenicity Test, Mutation Res., 31:347-364.

Maron, D.M. and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test, Mutation Res., 113:173-215.

Maron, D.M., J. Katzenellenbogen and B.N. Ames (1981) Compatibility of organic solvents with the Salmonella/Microsome Test, Mutation Res., 88:343-350.

Snee, R.D. and J.D. Irr (1981) Design of a statistical method for the analysis of mutagenesis at the hypoxanthine guanine phosphoribosyl transferase locus of cultured Chinese hamster ovary cells, Mutation Res., 85:77-93.

Table 1. Toxicity Prescreens

Compound	Dose**	Background Growth* TA1538	Background Growth* TA100
DMSO	100	+	+
MeNENA	50.0	+	+
	167	+	+
	500	+	+
	1670	+	+
	5000	+	+
EtNENA	50.0	+	+
	167	+	+
	500	+	+
	1670	+	+
	5000	+	+
BuNENA	50.0	+	+
	167	+	+
	500	+	+
	1670	±a/b	±a/b
	5000	±b	±b
DNPA/F -DPA	50.0	+	+
	167	+	+
	500[i]	+a	+a
	1670[i]	±a/b	±a/b
	5000[i]	±b	±b
DNPA/F +DPA	50.0	+	+
	167	+	+
	500[i]	+a/b	+a/b
	1670[i]	±a/b	±b
	5000[i]	±a/b	±b

*Background lawn characterized as normal (+) or inhibited (±)growth: a = slight toxicity; b = moderate toxicity.
**µl/plate.
[i]Test article incompletely soluble.

Table 2. Summary of Mutagenicity Results[a]

Test Article	Trial	Strain	±S9	Statistically Significant	Dose Dependent	Fold Increase
MeNENA	1	TA1535	-	yes	yes	1.6-fold
	1	TA1535	+	yes	yes	2.6-fold
	2	TA1535	-	yes	yes	2.0-fold
	2	TA1535	+	yes	yes	13-fold
	2	TA100	+	yes	yes	1.4-fold
EtNENA	1	TA1535	-	yes	yes	2.6-fold
	1	TA1535	+	yes	yes	19-fold
	1	TA98	+	yes	yes	1.6-fold
	1	TA100	+	yes	yes	3.1-fold
	2	TA1535	-	yes	yes	2.1-fold
	2	TA1535	+	yes	yes	20-fold
	2	TA100	+	yes	yes	1.3-fold
BuNENA	1	TA1535	-	yes	yes	1.8-fold
	1	TA1535	+	yes	yes	6.8-fold
	2	TA1535	+	yes	yes	3.5-fold
DNPA/F -DPA	1	TA1535	-	yes	yes	4.9-fold
	1	TA1535	+	yes	yes	5.9-fold
	1	TA98	+	yes	yes	1.7-fold
	1	TA100	-	yes	yes	1.5-fold
	1	TA100	+	yes	yes	1.5-fold
	2	TA1535	-	yes	yes	7.2-fold
	2	TA1535	+	yes	yes	9.0-fold
	2	TA100	-	yes	yes	1.7-fold
	2	TA100	+	yes	yes	1.7-fold
DNPA/F +DPA	1	TA1535	-	yes	yes	5.4-fold
	1	TA1535	+	yes	yes	6.8-fold
	1	TA98	-	yes	no	1.8-fold
	1	TA100	-	no	yes	1.4-fold
	1	TA100	+	yes	yes	1.6-fold
	2	TA1535	-	yes	yes	10-fold
	2	TA1535	+	yes	yes	8.9-fold
	2	TA100	-	yes	yes	1.5-fold
	2	TA100	+	yes	yes	1.7-fold

[a]Only statistically significant and/or dose-dependent increases reported; results for all other test article/strain/S9 combinations are considered to be negative (see Evaluation Criteria, pages 11-12).

Table 3. Comparison of Mutagenicity Results in Tester Strain TA1535 with S9

Test Article	Trial	Dose Range[a]	Number of Doses	Slope (rev per μmole/plate)[b]	Correlation Coefficient
MeNENA	1	0-1670	4	1.65	0.999
	2	0-5000	5	3.17	0.974
EtNENA	1	0-500	3	57.3	0.965
	2	0-500	3	42.8	1.00
BuNENA	1	0-500	3	35.0	0.925
	2	0-167	4	28.8	0.994
DNPA/F -DPA	1	0-1000	6	15.6	0.980
	2	0-1000	6	24.5	0.973
DNPA/F +DPA	1	0-1000	6	19.7	0.953
	2	0-1000	6	25.5	0.993

[a]μg/plate; initial linear portion of response.
[b]Revertants per μmole/plate; equal to (rev per μg/plate) x (molecular weight); slope and correlation coefficient calculated by simple linear regression on untransformed data.
[c]Average molecular weights calculated based upon weighted contribution from acetal and formal components (assumes no contribution from DPA stabilizer).

Table 4. Original Mutation Assay on Me-NENA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	15 (4)	10 (2)	3 (3)	21 (6)	100 (9)
DMSO (100 UL)		(+)	16 (2)	10 (3)	14 (2)	27 (1)	107 (18)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1356*(96)	--- (---)	--- (---)	--- (---)	1312*(114)
9-AMINOACRIDINE	150	(-)	--- (---)	1279*(38)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	471*(66)	408*(44)	--- (---)
2-ANTHRAMINE	2.50	(+)	117*(24)	604*(166)	1671*(133)	2298*(212)	2370*(195)
			TEST ARTICLE: Me-NENA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
167		(-)	14 (6)	9 (0)	5 (3)	18 (1)	85 (17)
500		(-)	17 (4)	8 (2)	3 (2)	17 (7)	100 (22)
1670		(-)	17 (5)	8 (2)	4 (2)	17 (3)	102 (24)
5000		(-)	21 (3)	10 (5)	4 (3)	20 (7)	100 (11)
7500		(-)	20 (5)	5 (1)	4 (1)	18 (3)	88 (22)
10000		(-)	24 (3)	7 (1)	3 (?)	18 (2)	99 (9)
167		(+)	17 (2)	12 (6)	14 (5)	30 (6)	103 (13)
500		(+)	21 (8)	14 (9)	13 (2)	22 (5)	121 (10)
1670		(+)	32*(9)	9 (3)	11 (6)	27 (2)	107 (15)
5000		(+)	38*(9)	5 (2)	14 (3)	23 (6)	121 (7)
7500		(+)	41*(3)	8 (4)	18 (2)	26 (4)	122 (25)
10000		(+)	37*(11)	6 (6)	8 (4)	24 (4)	97 (12)

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
Apparently normal growth all strains/doses +/-S9.
No precipitate.

USARMY/DT

Table 5. Confirmatory Mutation Assay on Me-NENA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	11 (4)	9 (7)	4 (3)	19 (2)	114 (3)
DMSO (100 UL)		(+)	9 (4)	7 (1)	12 (5)	24 (3)	131 (12)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1140*(62)	--- (---)	--- (---)	--- (---)	926*(133)
9-AMINOACRIDINE	150	(-)	--- (---)	1231*(55)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	273*(26)	287*(39)	--- (---)
2-ANTHRAMINE	2.50	(+)	137*(22)	603*(63)	1507*(130)	2173*(128)	2055*(239)
			TEST ARTICLE: Me-NENA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
167		(-)	8 (4)	4 (2)	3 (2)	18 (3)	113 (24)
500		(-)	9 (4)	3 (2)	1 (0)	11 (4)	110 (22)
1670		(-)	10 (4)	6 (3)	2 (1)	15 (2)	115 (3)
5000		(-)	14 (1)	4 (2)	2 (2)	16 (2)	108 (15)
7500		(-)	16 (4)	5 (3)	3 (3)	13 (1)	110 (16)
10000		(-)	23*(12)	3 (2)	2 (2)	14 (6)	106 (24)
167		(+)	15 (3)	5 (1)	15 (1)	32 (9)	138 (7)
500		(+)	34*(13)	6 (3)	8 (4)	24 (13)	154 (16)
1670		(+)	63*(32)	4 (1)	9 (4)	26 (10)	183 (31)
5000		(+)	109*(20)	6 (5)	11 (3)	17 (10)	182 (4)
7500		(+)	116*(43)	4 (1)	10 (4)	21 (8)	178 (37)
10000		(+)	66*(26)	7 (2)	11 (8)	23 (6)	168 (19)

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
Apparently normal growth all strains/doses +/- S9.
No precipitate.

USARMY/pt

Table 6. Original Mutation Assay on Et-NENA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	15 (4)	10 (2)	3 (3)	21 (6)	100 (9)
DMSO (100 UL)		(+)	16 (2)	10 (3)	14 (2)	27 (1)	107 (18)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1356‡(96)	--- (---)	--- (---)	--- (---)	1312‡(114)
9-AMINOACRIDINE	150	(-)	--- (---)	1279‡(38)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	471‡(66)	408‡(44)	--- (---)
2-ANTHRAMINE	2.50	(+)	117‡(24)	604‡(166)	1671‡(133)	2298‡(212)	2370‡(195)
			TEST ARTICLE: Et-NENA				
DOSE LEVEL	UG/PL	S-9	TA1335	TA1537	TA1538	TA98	TA100
167		(-)	14 (3)	12 (4)	6 (3)	21 (12)	97 (12)
500		(-)	16 (3)	12 (4)	7‡(5)	22 (3)	86 (11)
1670		(-)	22 (6)	7 (3)	6‡(3)	23 (7)	96 (6)
5000		(-)	36‡(5)	6 (3)	4 (1)	18 (2)	102 (10)a
7500		(-)	39‡(2)	5 (1)	4 (3)	21 (1)	119 (10)a
10000		(-)	39‡(7)	6 (1)	2 (1)	20 (10)a	113 (21)a
167		(+)	111‡(50)	15 (3)	14 (4)	44 (4)	149 (19)
500		(+)	184‡(81)	12 (5)	18 (7)	36 (11)	253‡(44)
1670		(+)	295‡(78)	12 (1)	22 (6)	43 (4)	335‡(79)
5000		(+)	223‡(102)	8 (2)	16 (5)a	28 (2)	251‡(12)a
7500		(+)	149‡(58)	9 (3)	17 (7)a/b	29 (1)	233‡(12)a
10000		(+)	82‡(8)	11 (4)	11 (6)a/b	32 (9)	197 (28)a/b

Data reported as: Mean (Standard Deviation).
‡Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
a/b = slight/moderate toxicity.
No precipitate.

USARMY/pt

Table 7. Confirmatory Mutation Assay on Et-NENA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	11 (4)	9 (7)	4 (3)	19 (2)	114 (3)
DMSO (100 UL)		(+)	9 (4)	7 (1)	12 (5)	24 (3)	131 (12)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1140$(62)	--- (---)	--- (---)	--- (---)	926$(133)
9-AMINOACRIDINE	150	(-)	--- (---)	1231$(55)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	273$(26)	287$(39)	--- (---)
2-ANTHRAMINE	2.50	(+)	137$(22)	603$(63)	1507$(130)	2173$(128)	2055$(239)
			TEST ARTICLE: Et-NENA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
167		(-)	12 (3)	5 (2)	2 (1)	19 (7)	120 (22)
500		(-)	15 (2)	4 (1)	1 (1)	19 (8)	103 (27)
1670		(-)	13 (6)	4 (2)	4 (2)	10 (7)	133 (17)
5000		(-)	16 (5)	4 (2)	2 (3)	11 (5)	106 (5)
7500		(-)	19 (1)	2 (1)	2 (1)	16 (1)	126 (6)
10000		(-)	24$(5)	3 (1)	0 (1)	13 (5)	118 (4)
167		(+)	46$(33)	5 (2)	11 (3)	26 (9)	143 (29)
500		(+)	128$(50)	9 (7)	11 (3)	25 (4)	133 (12)
1670		(+)	172$(63)	7 (3)	15 (2)	19 (5)	172 (25)
5000		(+)	153$(53)	5 (4)	7 (1)	27 (2)	165 (15)
7500		(+)	128$(27)	9 (5)	5 (2)	19 (7)	160 (22)
10000		(+)	82$(25)	6 (3)	2 (1)	16 (8)	119 (12)

Data reported as: Mean (Standard Deviation).
$Positive response (> 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
Apparently normal growth all strains/doses +/- S9.
No precipitate.

USARMY/pt

Ames/Salmonella Plate Incorporation
Assay on Five Unicharge Propellants
PH 301-US-001 ... 005-91

Table 8. Original Mutation Assay on Bu-NENA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	15 (4)	10 (2)	3 (3)	21 (6)	100 (9)
DMSO (100 UL)		(+)	16 (2)	10 (3)	14 (2)	27 (1)	107 (18)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1356*(96)	--- (---)	--- (---)	--- (---)	1312*(114)
9-AMINOACRIDINE	150	(-)	--- (---)	1279*(38)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	471*(66)	408*(44)	--- (---)
2-ANTHRAMINE	2.50	(+)	117*(24)	604*(166)	1671*(133)	2298*(212)	2370*(195)
			TEST ARTICLE: Bu-NENA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
50.0		(-)	11 (4)	11 (3)	5 (3)	23 (6)	90 (7)
167		(-)	15 (4)	7 (2)	6 (3)	16 (3)	97 (12)
500		(-)	21 (5)	5 (3)	6 (2)	20 (7)	102 (20)
1670		(-)	26 (10)a	8 (6)	5 (6)	16 (7)	87 (5)
3330		(-)	16 (3)b	2 (3)a	0 (1)a	10 (9)	86 (11)a
5000		(-)	7 (5)b/c	0 (1)a/b	0 (1)a/b	5 (1)a	13 (4)b/c
167		(+)	77*(11)	14 (6)	14 (5)	29 (10)	125 (21)
500		(+)	107*(19)a	10 (3)	15 (3)	21 (5)	129 (25)
1670		(+)	79*(6)a/b	9 (1)	13 (4)	26 (7)	136 (17)a
5000		(+)	15 (2)a/b	2 (1)a	2 (1)a	5 (2)a	27 (10)b
7500		(+)	6 (2)b/c	1 (1)a/b	0 (0)a/b	1 (1)b	7 (2)b
10000		(+)	1 (1)b/c	0 (1)a/b	2 (3)a/b	0 (0)b/c	0 (0)b/c

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
a/b/c = slight/moderate/severe toxicity.
No precipitate.

USARMY/pt

Table 9. Confirmatory Mutation Assay on Bu-NENA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	11 (4)	9 (7)	4 (3)	19 (2)	114 (3)
DMSO (100 UL)		(+)	9 (4)	7 (1)	12 (5)	24 (3)	131 (12)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1140*(62)	--- (---)	--- (---)	--- (---)	926*(133)
9-AMINOACRIDINE	150	(-)	--- (---)	1231*(55)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	273*(26)	287*(39)	--- (---)
2-ANTHRAMINE	2.50	(+)	137*(22)	603*(63)	1507*(130)	2173*(128)	2055*(239)
			TEST ARTICLE: Bu-NENA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
	16.7	(-)	14 (2)	9 (3)	4 (2)	13 (5)	108 (5)
	50.0	(-)	11 (6)	2 (2)	3 (1)	13 (6)	114 (11)
	167	(-)	8 (6)	6 (2)	2 (1)	12 (1)	99 (28)
	500	(-)	11 (2)	6 (1)	2 (2)a	12 (6)	103 (16)a
	1670	(-)	13 (2)	2 (2)a	2 (2)a/b	12 (3)a	102 (18)a
	5000	(-)	3 (1)a/b	1 (2)a/b	1 (2)a/b	2 (2)b/c	9 (1) b/c
	16.7	(+)	9 (5)	5 (1)	13 (6)	27 (3)	121 (12)
	50.0	(+)	13 (7)	9 (5)	8 (2)	23 (3)	116 (5)
	167	(+)	30*(6)	8 (6)	7 (4)	23 (4)	115 (5)
	500	(+)	25*(4)	5 (1)	8 (5)a	19 (5)	128 (28)
	1670	(+)	17 (4)a/b	7 (2)a	7 (2)a/b	20 (1)a	103 (5)a
	5000	(+)	2 (1)b/c	1 (1)a/b	1 (1)b/c	1 (1)a/b	4 (2)b/c

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
a/b/c = slight/moderate/severe toxicity.
No precipitate.

USARMY/pt

Table 10. Original Mutation Assay on DNPA/F -DPA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	10 (3)	7 (2)	3 (2)	11 (3)	80 (14)
DMSO (100 UL)		(+)	13 (4)	7 (2)	9 (5)	18 (3)	89 (11)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1006*(77)	--- (---)	--- (---)	--- (---)	784*(32)
9-AMINOACRIDINE	150	(-)	--- (---)	1024*(35)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	224*(36)	363*(41)	--- (---)
2-ANTHRAMINE	2.50	(+)	239*(60)	499*(137)	1684*(83)	1979*(56)	2127*(93)
			TEST ARTICLE: Bis-DNPA/F -DPA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
16.7		(-)	16 (3)	7 (1)	2 (2)	14 (1)	99 (3)
50.0		(-)	17 (1)	6 (3)	2 (1)	10 (4)	118 (9)
167		(-)	21*(5)	5 (2)	3 (3)	11 (4)	115 (8)
500		(-)	40*(4)	7 (2)	2 (1)	12 (3)	93 (17)
1000		(-)	50*(6)a/b	3 (3)a/b	2 (1)	16 (5)a	35 (8)a
1670		(-)	42*(3)a/b	3 (0)b/c	1 (1)a	9 (3)a/b	14 (7)b/c
16.7		(+)	15*(6)	6 (2)	10 (4)	31 (7)	126 (31)
50.0		(+)	16 (1)	7 (2)	6 (2)	17 (3)	108 (4)
167		(+)	30*(5)	4 (1)	4 (1)	19 (5)	136 (12)
500		(+)	44*(1)	5 (2)	10 (3)	19 (2)	136 (9)
1000		(+)	62*(15)a	3 (1)	6 (3)	22 (6)	68 (13)
1670		(+)	74*(11)a/b	3 (2)a/b	3 (2)	17 (4)a/b	21 (10)a/b

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated;
other statistically significant increases are indicated in Table 2).
a/b/c = slight/moderate/severe toxicity.
Test article droplets at ≥500 ug/plate.

USARMY/pt

Ames/Salmonella Plate Incorporation
Assay on Five Unicharge Propellants
PH 301-US-001 ... 005-91

Table 11. Confirmatory Mutation Assay on DNPA/F -DPA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	9 (1)	6 (3)	3 (2)	22 (5)	80 (22)
DMSO (100 UL)		(+)	10 (3)	8 (3)	10 (4)	30 (3)	92 (9)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1296*(115)	--- (---)	--- (---)	--- (---)	1312*(77)
9-AMINOACRIDINE	150	(-)	--- (---)	1239*(100)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	163*(17)	323*(66)	--- (---)
2-ANTHRAMINE	2.50	(+)	172*(25)	597*(63)	1805*(64)	2514*(228)	2400*(292)
			TEST ARTICLE: Bis-DNPA/F -DPA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
16.7		(-)	13 (3)	9 (5)	2 (2)	19 (5)	100 (12)
50.0		(-)	21*(10)	5 (2)	1 (1)	14 (7)	103 (8)
167		(-)	26*(10)	13*(4)	3 (3)	20 (8)	132 (9)
500		(-)	55*(7)	4 (1)	1 (2)	18 (10)	123 (23)
1000		(-)	63*(15)a/b	5 (3)a/b	2 (2)	21 (4)a	93 (17)a/b
1670		(-)	62*(12)a/b	1 (1)b	2 (2)a	21 (4)a	38 (20)b
16.7		(+)	18 (2)	13 (5)	13 (1)	24 (6)	98 (8)
50.0		(+)	22*(8)	6 (3)	8 (4)	22 (4)	105 (17)
167		(+)	36*(6)	8 (2)	7 (3)	21 (5)	135 (11)
500		(+)	69*(8)	4 (2)	8 (2)	24 (1)	156 (29)
1000		(+)	89*(7)a	4 (2)	14 (5)	26 (5)	96 (7)
1670		(+)	93*(9)a/b	4 (4)a/b	6 (4)a	25 (6)a	55 (18)a/b

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated;
other statistically significant increases are indicated in Table 2).
a/b = slight/moderate toxicity.
Test article droplets at ≥500 ug/plate.

USARMY/pt

Table 12. Original Mutation Assay on DNPA/F +DPA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	10 (3)	7 (2)	3 (2)	11 (3)	8ᵕ (14)
DMSO (100 UL)		(+)	13 (4)	7 (2)	9 (5)	18 (3)	89 (11)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1006$(77)	--- (---)	--- (---)	--- (---)	784$(32)
9-AMINOACRIDINE	150	(-)	--- (---)	1024$(35)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	224$(36)	363$(41)	--- (---)
2-ANTHRAMINE	2.50	(+)	239$(60)	499$(137)	1684$(83)	1979$(56)	2127$(93)
			TEST ARTICLE: Bis-DNPA/F +DPA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
16.7		(-)	10 (3)	8 (0)	3 (2)	21 (8)	99 (13)
50.0		(-)	17 (2)	5 (1)	2 (1)	19 (6)	108 (19)
167		(-)	31$(7)	4 (2)	4 (2)	15 (5)	107 (40)
500		(-)	48$(5)	5 (2)a	4 (4)a	15 (5)	107 (17)a
1000		(-)	53$(11)a	2 (1)b	3 (1)a/b	15 (2)a	53 (15)b
1670		(-)	56$(9)a/b	3 (1)b	1 (2)a/b	10 (4)b	17 (4)b
16.7		(+)	12 (4)	12 (10)	13 (1)	20 (5)	115 (17)
50.0		(+)	26 (4)	7 (2)	10 (4)	26 (3)	124 (4)
167		(+)	27$(5)	9 (5)	8 (5)	17 (2)	122 (8)
500		(+)	64$(11)a	5 (3)	6 (3)	22 (7)	147 (12)
1000		(+)	74$(10)a/b	4 (3)a	4 (3)a	15 (4)a/b	100 (8)a/b
1670		(+)	86$(6)a/b	3 (1)a/b	4 (2)a	22 (6)a/b	40 (13)a/b

Data reported as: Mean (Standard Deviation).
$Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
a/b = slight/moderate toxicity.
Test article droplets at ≥500 ug/plate.

USARMY/pt

Ames/Salmonella Plate Incorporation
Assay on Five Unicharge Propellants
PH 301-US-001 ... 005-91

Table 13. Confirmatory Mutation Assay on DNPA/F +DPA

			CONTROLS				
			AVERAGE REVERTANTS/PLATE				
SOLVENT CONTROLS		S-9	TA1535	TA1537	TA1538	TA98	TA100
DMSO (100 UL)		(-)	9 (1)	6 (3)	3 (2)	22 (5)	80 (22)
DMSO (100 UL)		(+)	10 (3)	8 (3)	10 (4)	30 (3)	92 (9)
POSITIVE CONTROLS	UG/PL						
SODIUM AZIDE	10.0	(-)	1296*(115)	--- (- -)	--- (---)	--- (---)	1312*(77)
9-AMINOACRIDINE	150	(-)	--- (---)	1239*(100)	--- (---)	--- (---)	--- (---)
2-NITROFLUORENE	5.00	(-)	--- (---)	--- (---)	163*(17)	323*(66)	--- (---)
2-ANTHRAMINE	2.50	(+)	172*(25)	597*(63)	1805*(64)	2514*(228)	2400*(292)
			TEST ARTICLE: Bis-DNPA/F +DPA				
DOSE LEVEL	UG/PL	S-9	TA1535	TA1537	TA1538	TA98	TA100
	16.7	(-)	14 (3)	10 (6)	4 (2)	16 (0)	92 (9)
	50.0	(-)	16 (2)	15*(3)	3 (1)	14 (4)	106 (21)
	167	(-)	29*(3)	7 (6)	4 (2)	19 (5)	117 (17)
	500	(-)	44*(7)	3 (1)a	2 (1)a	22 (7)	122 (15)a
	1000	(-)	58*(8)a	4 (1)a/b	2 (2)a/b	20 (5)a	80 (6)a/b
	1670	(-)	88*(12)a	3 (3)a/b	2 (2)a/b	25 (5)a/b	38 (2)a/b
	16.7	(+)	14 (2)	9 (2)	12 (2)	28 (3)	111 (10)
	50.0	(+)	20 (5)	9 (3)	6 (3)	28 (12)	102 (9)
	167	(+)	30*(9)	7 (3)	7 (1)	23 (5)	144 (16)
	500	(+)	61*(16)	8 (3)	9 (2)	23 (4)	155 (6)
	1000	(+)	92*(18)a	6 (1)a/b	10 (4)a/b	26 (5)a/b	116 (19)a
	1670	(+)	92*(9)a	6 (3)a/b	11 (1)a/b	31 (2)a/b	61 (9)a/b

Data reported as: Mean (Standard Deviation).
*Positive response (≥ 2X solvent control value. Only two-fold or greater increases are indicated; other statistically significant increases are indicated in Table 2).
a/b = slight/moderate toxicity.
Test article droplets at ≥500 ug/plate.

USARMY/pt

COMPLIANCE STATEMENT

Except that analytical analyses of dosing solutions were not performed to verify the accuracy or stability of the test article dosing solutions, this study was conducted in compliance with the Principles of Good Laboratory Practices (GLP) as promulgated by the following regulatory agencies:

U.S. Food and Drug Administration, as stated in the Federal Register, 21 CFR Part 58.

U.S. Environmental Protection Agency as stated in the Federal Register, 40 CFR Parts 160 and 792.

Organization for Economic Co-operation and Development Guidelines for Testing Chemicals (OECD), ISBN 92-64-12221-4.

Study Nos.: PH 301-US-001-91
PH 301-US-002-91
PH 301-US-003-91
PH 301-US-004-91
PH 301-US-005-91

"To the best of my knowledge, this study was conducted in accordance with applicable Good Laboratory Practice regulations except as noted above; there were no other deviations from these regulations that impacted on study conclusions."

Study Director

29 MAY 1992
Date

QUALITY ASSURANCE UNIT STATEMENT

Study Nos.: PH 301-US-001-91
PH 301-US-002-91
PH 301-US-003-91
PH 301-US-004-91
PH 301-US-005-91

Study Director: Leon F. Stankowski, Jr., Ph.D.

The Quality Assurance Unit conducted the inspections listed below and reported the results to the study director and to management on the dates indicated.

The following inspections were performed:

Interval	Date
Plating Phase	October 9, 1991
	October 11, 1991
	October 21, 1991
	October 22, 1991
Scoring Phase	October 11, 1991
	October 14, 1991
Reporting Phase	November 22, 1991

Date QAU Report Issued

To Study Director	To Management
November 22, 1991	November 22, 1991

Quality Assurance

May 29 1992
Date